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STEROLS, STEROL ESTERS AND FATTY ACIDS OF *BOTRYDIUM GRANULATUM*, *TRIBONEMA AEQUALE* AND *MONODUS SUBTERRANEUS*

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Key Word Index—*Botrydium granulatum*; *Tribonema aequale*; *Monodus subterraneus*; Xanthophyceae; sterols; cholesterol; clionasterol; cycloartenol; 24-methylenecycloartanol; C_{16} fatty acids.

Abstract—The composition of the sterols, sterol esters and fatty acids has been determined in 8-, 11- and 14-day cultures of three members of the Xanthophyceae, *Botrydium granulatum*, *Tribonema aequale* and *Monodus subterraneus*. The main sterols, whether esterified or unesterified, were cholesterol and clionasterol, whose proportions do not vary with age of culture. Much smaller quantities of cycloartenol and 24-methylenecycloartanol were also found in all three algae. The C_{16} fatty acids are the most common fatty acids in all three algae with $C_{16:1}$ being particularly abundant. *B. granulatum* and *T. aequale*, however, differ from *M. subterraneus* in having polyunsaturated C_{16} fatty acids and a smaller proportion of $C_{20:5}$.

INTRODUCTION

THE STEROLS of algae appear to be far more varied than those found in higher plants. The major sterols of the red algae (Rhodophyta) are C_{27} sterols; most of the species examined have cholesterol as the predominant sterol although several species contain large amounts of desmosterol.[1-7] Fucosterol is the major sterol of the brown algae (Phaeophyta) with

Abbreviations. The trival names of the sterols used in the text have the following systematic names: cholesterol = cholest-5-en-3β-ol; desmosterol = cholesta-5,24-dien-3β-ol; 24-methylenecholesterol = 24-methylenecholest-5-en-3β-ol; Δ^7-ergostenol = (24*S*)-24-methylcholest-7-en-3β-ol; 22-dihydrobrassicasterol = (24*S*)-24-methylcholest-5-en-3β-ol; brassicasterol = (24*R*)-24-methylcholesta-5,22-dien-3β-ol; ergosterol = (24*R*)-24-methylcholesta-5,7,22-trien-3β-ol; fucosterol = *E*-24-ethylidenecholest-5-en-3β-ol; 28-isofucosterol = *Z*-24-ethylidenecholest-5-en-3β-ol; sitosterol = (24*R*)-24-ethylcholest-5-en-3β-ol; clionasterol = (24*S*)-24-ethylcholest-5-en-3β-ol; Δ^7-chondrillasterol = (24*S*)-24-ethylcholest-7-en-3β-ol; chondrillasterol = (24*R*)-24-ethylcholesta-7,-22-dien-3β-ol; poriferasterol = (24*R*)-24-ethylcholesta-5,22-dien-3β-ol; lanosterol = 4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol; cycloartenol = 4,4,14α-trimethyl-9,19-cyclo-5α-cholest-24-en-3β-ol; 24-methylenecycloartanol = 24-methylene-4,4,14α-trimethyl-9,19-cyclo-5α-cholestan-3β-ol. (Note that in saturated side chains 24α- and 24β-alkyl substituents become (24*R*)-and (24*S*)-24-alkyl substituents respectively according to the Cahn, Ingold and Prelog convention;[30] however, the presence of a Δ^{22}-double bond reverses the specification of chirality at C-24[31].)

[1] TSUDA, K., AKAGI, S. and KISHIDA, Y. (1957) *Science* **126**, 927.
[2] TSUDA, K., AKAGI, S. and KISHIDA, Y. (1958) *Chem. Pharm. Bull. (Tokyo)* **6**, 101.
[3] TSUDA, K., AKAGI, S., KISHIDA, Y., HAYATSU, R. and SAKAI, K. (1958) *Chem. Pharm. Bull. (Tokyo)* **6**, 724.
[4] AARONSON, S. and BAKER, H. (1961) *J. Protozool.* **8**, 274.
[5] GIBBONS, G. F., GOAD, L. J. and GOODWIN, T. W. (1967) *Phytochemistry* **6**, 677.
[6] ALCAIDE, A., DEVYS, M. and BARBIER, M. (1968) *Phytochemistry* **7**, 329.
[7] IDLER, D. R., SAITO, A. and WISEMAN, P. (1968) *Steroids* **11**, 465.
[30] CAHN, R. S., INGOLD, C. K. and PRELOG, V. (1956) *Experientia* **12**, 81.
[31] BROOKS, C. J. W. (1970) *Rodd's Chemistry of Carbon Compounds* (COFFEY, S., ed.), Vol. IID, p. 75, Elsevier, New York.

some species also having 24-methylenecholesterol.[8–13] The sterols of green algae (Chlorophyta) are much more varied than those of other divisions of algae and many species have very complex mixtures of sterols. Fourteen species of the genus *Chlorella* have been studied in detail and shown to vary markedly in sterol composition; seven species contain ergosterol and other $\Delta^{5,7}$-sterols, five species contain Δ^{7}-ergosterol, chondrillasterol and Δ^{7}-chondrillasterol and two species contain dihydrobrassicasterol, poriferasterol and clionasterol.[14–16] Some species of Chlorophyta have significant amounts of cholesterol,[12] 24-methylenecholesterol[12] and 28-isofucosterol.[17,18] There is considerable evidence to indicate that methyl or ethyl substituents on C-24 of the sterols of Chlorophyta have the β-orientation (24*S*) in contrast to the α-orientation (24*R*) of their counterparts in higher plants.[16] Small amounts of sterols have been isolated from two blue–green algae (Cyanophyta), 24-ethylcholesterol and 24-ethyl-$\Delta^{7,22}$-cholestadienol from *Phormidium luridum*[19] and cholesterol and 24-ethylcholesterol from *Anacystis nidulans* and *Fremyella diplosiphon*.[20] The configuration at C-24 in these sterols remains to be decided. *Euglena gracilis* (Euglenophyta) has been shown to contain ergosterol.[21,22]

In the Chrysophyta most of the recent sterol investigations utilizing such powerful analytical tools as GLC and MS have been carried out on a few species of golden-brown algae (Chrysophyceae). *Synura petersenii* has been shown to possess cholesterol and sitosterol.[23] *Ochromonas danica* contains ergosterol, brassicasterol, 22-dihydrobrassicasterol, clionasterol, poriferasterol and probably 7-dehydroporiferasterol.[24] *Ochromonas malhamensis*, on the other hand, contains only poriferasterol as the major sterol component.[24] The sterol content of the other classes of the Chrysophyta, the yellow–green algae (Xanthophyceae) and the diatoms (Bacillariophyceae) has not been recently investigated. Accordingly we have carried out such an investigation on members of three orders of Xanthophyceae, *Botrydium granulatum* (Heterosiphonales), *Tribonema aequale* (Heterotrichales) and *Monodus subterraneus* (Heterococcales). We have determined not only the total sterol composition but also the composition of the sterols present as sterol esters in cultures of these algae grown for different lengths of time so as to check for variations with age of culture. We have also determined the composition of the fatty acids derived from the sterol esters and from the total lipid to check for differences which could be indicative of specificity with respect to the fatty acid content of the sterol esters.

[8] HEILBRON, I. M. (1942) *J. Chem. Soc.* 79.
[9] BLACK, W. A. P. and CORNHILL, W. J. (1951) *J. Sci. Food. Agr.* **2**, 387.
[10] TSUDA, K., HAYATSU, R., KISHIDA, Y. and AKAGI, S. (1958) *J. Am. Chem. Soc.* **80**, 921.
[11] IKEKAWA, N., TSUDA, K. and MORISAKI, N. (1966) *Chem. Ind.* 1179.
[12] IKEKAWA, N., MORISAKI, N., TSUDA, K. and YOSHIDA, T. (1968) *Steroids* **12**, 41.
[13] CIERESZKO, L. S., JOHNSON, M. A., SCHMIDT, R. W. and KOONS, C. B. (1968) *Comp. Biochem. Physiol.* **24**, 899.
[14] PATTERSON, G. W. (1967) *Plant Physiol.* **42**, 1457.
[15] PATTERSON, G. W. (1969) *Comp. Biochem. Physiol.* **31**, 391.
[16] PATTERSON, G. W. (1971) *Lipids* **6**, 120.
[17] TSUDA, K. and SAKAI, K. (1960) *Chem. Pharm. Bull.* (*Tokyo*) **8**, 554.
[18] GIBBONS, G. F., GOAD, L. J. and GOODWIN, T. W. (1968) *Phytochemistry* **7**, 983.
[19] REITZ, R. C. and HAMILTON, J. G. (1968) *Comp. Biochem. Physiol.* **25**, 401.
[20] DE SOUZA, N. J. and NES, W. R. (1968) *Science* **162**, 363.
[21] STERN, A. I., SCHIFF, J. A. and KLEIN, H. P. (1960) *J. Protozool.* **7**, 52.
[22] AVIVI, L., IARON, O. and HALEVY, S. (1967) *Comp. Biochem. Physiol.* **21**, 321.
[23] COLLINS, R. P. and KALNINS, K. (1969) *Comp. Biochem. Physiol.* **30**, 779.
[24] GERSHENGORN, M. C., SMITH, A. R. H., GOULSTON, G., GOAD, L. J., GOODWIN, T. W. and HAINES, T. H. (1968) *Biochemistry* **7**, 1698.

RESULTS

Thirteen 1-litre batches of Bold's Basal Medium were inoculated with equal vols of a logarithmic-phase culture of *B. granulatum*. After 8, 11 and 14 days of growth the cells from 5, 4 and 4 l. were harvested and the lipid extracted, yielding 72·9, 107·5 and 160·6 mg respectively. *T. aequale* was treated in the same way and yielded 63·5, 95·6 and 147·5 mg of lipid from 5 l. of 8 day, 4 l. of 11 day and 4 l. of 14 day cultures respectively. Nine 1-litre batches of *M. subterraneus* were grown in shake culture like the other two algae; 3 l. harvested after 8 days yielded 43·5 mg of lipid whilst two batches of 3 l. harvested after 11 and 14 days yielded 47·9 and 82·8 mg respectively. Thirteen 1-litre batches of *M. subterraneus* were also grown with forced aeration; 5 l. harvested after 8 days yielded 213·2 mg of lipid whilst two batches of 4 l. harvested after 11 and 14 days yielded 301·6 and 423·4 mg respectively.

The lipid samples were normally divided into two parts, one representing 2/3rd of the total and one representing 1/3rd. The 2/3rd portions were saponified and the unsaponifiable material and fatty acids isolated. The fatty acids were methylated, purified by TLC (system 2) and analysed by GLC before and after hydrogenation. The unsaponifiable material was fractionated by column chromatography. The fractions were checked for sterol content by TLC (system 1) and those containing sterol bulked. The 4-demethylsterols, co-chromatographing with cholesterol and the 4,4-dimethylsterols, co-chromatographing with lanosterol, were then separated from the bulked fractions by TLC (system 1). The sterols in the 4,4-dimethylsterol zone constituted 1–3% of the total sterols of the algae. The 4-demethyl- and 4,4-dimethylsterols were then analysed by GLC and GC–MS. The 1/3rd portions of lipids were used for sterol ester analysis. The sterol esters were isolated by column chromatography and purified by TLC (system 2). They were then saponified and the resulting sterols and fatty acids extracted and analysed in the same way as the sterols and fatty acids obtained from the total lipid. The quantities of lipid obtained from the 8 and 11 day cultures of *B. granulatum* and *T. aequale* and the shake cultures of *M. subterraneus* were considered too small to be divided into two parts and were therefore analysed in the same way as the 2/3rd portions of lipid described above.

GLC analysis of the 4-demethylsterols derived from the total lipid and the sterol esters of all three algae revealed two components, A and B, which co-chromatographed with cholesterol and sitosterol respectively. The MS of sterol A (Table 1) had a molecular ion at *m/e* 386 suggesting a C_{27} sterol with one double bond. Ions *d* and *e* at *m/e* values of 273 and 271 showed that the side chain was C_8H_{17} and that the double bond was in the nucleus. Ions *v*, *x* and *y* at *m/e* values of 301, 275 and 247 strongly suggest[25] that the position of the double bond is Δ^5. This evidence is therefore consistent with the identification of sterol A as cholesterol. The mass spectrum of sterol B (Table 1) had a molecular ion at *m/e* 414 suggesting a C_{29} sterol with one double bond. Ions *d* and *e* at *m/e* values of 273 and 271 showed that the side chain was $C_{10}H_{21}$ and that the double bond was in the nucleus. Ions *v*, *x* and *y* at *m/e* values of 329, 303 and 275 strongly suggest [25] that the position of the double bond is Δ^5. This evidence indicates that sterol B is a 24-ethylcholesterol. The orientation of the ethyl group at C-24 was shown to be β (*S* according to the Cahn, Ingold and Prelog convention)[30] by optical rotation measurements. The specific rotations $[\alpha]_D^{25}$ in $CHCl_3$ of sterol B derived from all three algae were found to be in the range $-42·5°$ to $-44·5°$. The literature values for the $[\alpha]_D^{25}$ in $CHCl_3$ of 24α-ethylcholesterol (sitosterol)

[25] Knights, B. A. (1967) *J. Gas. Chromatogr.* 5, 273.

TABLE 1. IONIC SPECIES IN THE MS OF THE STEROLS OF *Botrydium granulatum*, *Tribonema aequale* AND *Monodus subterraneus*
(Intensities of the ions are shown in parenthesis*)

Ion	Fragmentation	Sterol A	B	C	D
M^+	Molecular ion	386 (53)	414 (53)	426 (22)	440 (24)
a	M^+—Me	371 (51)	399 (41)	411 (63)	425 (60)
b	M^+—HOH	368 (80)	396 (71)	408 (37)	422 (36)
c	M^+—[Me + HOH]	353 (73)	381 (65)	493 (100)	407 (100)
d	M^+—SC	273 (27)	273 (32)	315 (13)	315 (20)
e	M^+—[SC + 2H]	271 (8)	271 (9)	313 (19)	313 (32)
f	M^+—[SC + HOH]	255 (61)	255 (65)	297 (26)	297 (40)
g	M^+—[SC + HOH + 2H]	253 (6)	253 (5)	295 (39)	295 (100)
h	M^+—[SC + 27]	246 (12)	246 (11)	288 (7)	288 (16)
i	M^+—[SC + 27 + HOH]	228 (24)	228 (26)	270 (9)	270 (24)
j	M^+—[SC + 42]	231 (41)	231 (41)	273 (19)	273 (32)
k	M^+—[SC + 42 + HOH]	213 (100)	213 (100)	255 (24)	255 (32)
l	M^+—[84]				
m	M^+—[84 + Me]				
n	M^+—[84 + Me + HOH]				
o	M^+—[43]				
p	M^+—[43 + HOH]				
q	M^+—[C − 22→ +H]				
r	M^+—[C − 22→ +H + Me]				
s	M^+—[SC + 56]			259 (31)	259 (40)
t	M^+—[SC + 56 + HOH]			241 (22)	241 (44)
u	M^+—[59]				
v	M^+—[67 + HOH]	301 (68)	329 (53)		
x	M^+—[93 + HOH]	275 (100)	303 (82)		
y	M^+—[121 + HOH]	247 (60)	275 (29)		

* Intensities are taken from the MS of the sterols from the 14 day *B. granulatum* culture; however, similar values were obtained from the MS of the equivalent sterols from other algae.

SC = side chain: 27 = [C-16 + C-17 + 3H]: 42 = [C-15 + C-16 + C-17 + 6H]: 84 = [C-23 to C-28] in 24-methylene side chains of sterols other than those with a 9,19-cyclopropane ring; 43 = [C-24 to C-27] in Δ^{22} side chains: [C-22→ +H] = H plus C-22 to the end of SC: occurs only in the presence of Δ^{22} unsaturation: 56 = [C-15 to C-17 + C-32 + 8H]: 56 = [C-1 to C-3 + OH + 6H]; characteristic of $\Delta^{5,7}$-sterols: 67 = C_5H_7 from C-2 to C-6 or C-3 to C-7 in Δ^5-sterols: 93 = C_7H_9 from Rings A and B, probably C-1 to C-7, in Δ^5-sterols: 121 = C_9H_{13} from Rings A and B by cleavage of the C-7 to C-8 and C-9 to C-10 bonds.

and 24β-ethylcholesterol (clionasterol) are $-37°$ and $-42°$. Therefore sterol B is identified as clionasterol.

GLC analysis of the small quantities of 4,4-dimethylsterols derived from the total lipid and sterol ester of the three algae revealed several components, two of which (C and D, Table 1) had the same RR_T* values as cycloartenol and 24-methylenecycloartanol. The mass spectrum of sterol C (Table 1) had a molecular ion at *m/e* 426, suggesting a C_{30} sterol with either two double bonds or one double bond and a 9,19-cyclopropane ring. Ions *d* and *e* at *m/e* values of 315 and 313 showed that the side chain was C_8H_{15}. Lack of ions *q* and *r* at *m/e* values of 342 and 327 show that the double bond in the side chain is not at Δ^{22} and point to a Δ^{24} location. Ions *s* and *t* at *m/e* values of 259 and 241 indicate the presence of a 14α-methyl group.[26] This MS evidence plus the fact that sterol C had the same RR_t as cycloartenol and clearly separated from authentic lanosterol on GLC, identi-

* Retention time relative to cholestane.

[26] GOAD, L. J. and GOODWIN, T. W. (1967) *European J. Biochem.* **1**, 357.

TABLE 2. COMPOSITION OF THE 4-DEMETHYL STEROLS ISOLATED AFTER SAPONIFICATION OF THE TOTAL LIPID OF *Botrydium granulatum*, *Tribonema aequale* AND *Monodus subterraneus* GROWN FOR 8, 11 AND 14 DAYS IN LIQUID CULTURE

Alga	Sterol	Percentage composition* 8 day	11 day	14 day
Botrydium	Cholesterol	14·1	15·2	14·0
	Clionasterol	85·9	84·8	86·0
Tribonema	Cholesterol	30·9	33·2	31·6
	Clionasterol	69·1	66·8	68·4
Monodus	Cholesterol	32·6 (40·0)†	33·7 (41·1)†	33·5 (39·0)†
	Clionasterol	67·4 (60·0)	66·3 (58·9)	66·5 (61·0)

* Based on peak areas from GLC on 3% OV-1.

† Figures in parenthesis refer to percentage composition in *M. subterraneus* cultures grown with forced aeration; all other figures refer to algae grown in shake culture.

fies it as cycloartenol. The mass spectrum of sterol D had a molecular ion at *m/e* 440 suggesting a C_{31} sterol with either two double bonds or one double bond and a 9,19-cyclopropane ring. Ions *d* and *e* at *m/e* values of 315 and 313 showed that the side chain was C_9H_{17}. Lack of ions *q* and *r* at *m/e* values of 342 and 327 show that the double bond in the side chain is not Δ^{22} and point to the presence of a 24-methylene group. Ions *s* and *t* at *m/e* values of 259 and 241 indicate the presence of a 14α-methyl group.[26] Lack of ions *l*, *m* and *n* at *m/e* values of 356, 341 and 323 respectively indicate the presence of a 9,19-cyclopropane ring. Ions *l*, *m* and *n* are characteristic of 24-methylenesterols provided they do not contain a 9,19-cyclopropane ring. The evidence therefore indicates that sterol D is 24-methylenecycloartanol.

The percentage of the two most abundant sterols, the 4-demethylsterols cholesterol and clionasterol, in the total lipid of the three algae grown for 8, 11 and 14 days is shown in Table 2. The ratios of these two sterols in each alga does not change with age of culture. In *B. granulatum* cholesterol and clionasterol constitute about 15% and 85% of the 4-demethylsterols present in the lipid respectively where as in *T. aequale* and the shake culture of *M. subterraneus* they constitute about 32% and 68% respectively. However, the cultures of *M. subterraneus* grown with forced aeration have an increased percentage of cholesterol (~40%).

The percentage of cholesterol and clionasterol in the sterol esters of the three algae is shown in Table 3. In *M. subterraneus* the ratio of the two sterols does not change with age of culture but the proportion of cholesterol is slightly lower than in the total lipid. The proportion of cholesterol in the sterol esters from the 14 day culture of *B. granulatum*

TABLE 3. COMPOSITION OF THE 4-DEMETHYLSTEROLS OBTAINED AFTER SAPONIFICATION OF THE STEROL ESTERS OF *Botrydium granulatum*, *Tribonema aequale* AND *Monodus subterraneus*

	Percentage composition*				
	Botrydium	*Tribonema*		*Monodus*†	
Sterol	14 day	14 day	8 day	11 day	14 day
Cholesterol	18·3	29·7	38·4	36·9	36·5
Clionasterol	81·7	70·3	61·6	63·1	63·5

* Based on peak areas from GLC on 3% OV-1.

† *Monodus* was grown on Bold's Basal Medium with forced aeration; *Botrydium* and *Tribonema* were grown in shake culture on the same medium.

TABLE 4. COMPOSITION OF THE FATTY ACIDS OBTAINED AFTER SAPONIFICATION OF THE TOTAL LIPID OF *Botrydium granulatum*, *Tribonema aequale* AND *Monodus subterraneus* GROWN FOR 8, 11 AND 14 DAYS IN LIQUID CULTURE

	Percentage composition											
Fatty	*Botrydium*			*Tribonema*			*Monodus*					
acid*	8[1]	11[1]	14[1]	8[1]	11[1]	14[1]	8[1]	11[1]	14[1]	8[2]	11[2]	14[2]
10:0	0·3	1·3	0·2	0·0	0·0	0·0	2·1	0·2	1·0	0·3	0·8	0·3
12:0	0·9	1·3	0·3	0·7	0·1	0·2	9·5	0·8	7·9	1·5	2·4	0·5
14:0	4·8	4·3	2·6	9·7	7·5	8·0	8·4	4·1	9·1	5·5	8·8	3·4
14:1	2·2	1·5	0·3	1·3	0·5	0·3	1·3	0·0	0·8	0·0	0·0	0·0
15:0	0·4	0·5	0·4	0·6	0·4	0·4	1·2	0·3	0·5	0·6	0·4	0·3
16:0	9·5	5·6	21·2	10·9	25·8	21·1	13·2	21·2	10·2	15·0	10·9	17·7
16:1	29·1	29·1	50·6	42·0	49·2	48·9	30·2	37·4	33·4	31·2	37·8	37·9
16:?†	3·3	3·3	1·2	1·5	0·8	0·9	0·0	0·0	0·0	0·0	0·0	0·0
16:2	14·4	17·4	6·1	5·6	2·4	2·7	0·0	0·0	0·0	0·0	0·0	0·0
16:3	26·2	29·7	6·1	9·4	2·0	2·2	0·0	0·0	0·0	0·0	0·0	0·0
17:0	0·0	0·0	0·0	0·0	0·0	0·0	1·9	0·3	0·5	1·0	1·7	0·5
18:0	0·9	0·7	0·4	0·9	0·6	0·6	1·2	1·0	0·8	0·6	0·9	0·3
18:1	0·3	0·4	0·9	1·3	1·1	1·7	4·1	7·1	2·8	4·6	2·6	2·6
18:2	0·8	0·6	1·3	1·3	1·2	1·3	4·6	4·3	3·6	3·0	2·6	1·5
18:3	0·5	0·4	0·4	0·6	0·1	0·2	2·8	0·4	0·7	0·9	0·8	0·4
20:0	0·3	0·1	0·0	0·3	0·1	0·1	1·7	0·0	1·9	0·3	0·6	0·3
20:4	1·5	0·6	3·4	3·9	4·3	5·9	2·9	1·7	1·0	4·6	1·5	2·3
20:5	4·4	3·3	4·7	10·2	3·7	5·6	14·9	21·3	25·9	30·9	28·2	32·0

The fatty acids were analysed as their methyl esters on either 10% SP 1000 or 10% FFAP.

* The number before the colon is the number of carbon atoms in the fatty acid; the number after the colon is the number of double bonds present.

† This minor fatty acid chromatographed as its methyl ester between those of $C_{16:1}$ and $C_{16:2}$; however, it disappeared from the GLC trace after hydrogenation.

(1) Algae were grown for 8, 11 or 14 days on Bold's Basal Medium at 23° on a gyrotary shaker under constant illumination.

(2) Algae were grown for 8, 11 or 14 days on Bold's Basal Medium at 20° under constant illumination and with forced aeration.

is slightly higher than that in the total lipid whilst the reverse was the case in *T. aequale*.

The ratios of cycloartenol to 24-methylenecycloartenol in the 4,4-dimethylsterol fractions derived from the total lipid and sterol esters of *B. granulatum*, *T. aequale* and *M. subterraneus* were in the order of 2:1, 2:1 and 6:1 respectively.

The composition of the fatty acids obtained after saponification of the total lipid of the three algae grown for 8, 11 and 14 days is shown in Table 4. The C_{16} fatty acids are the most common fatty acids in all three algae with $C_{16:1}$ being particularly abundant. *B. granulatum* and *T. aequale*, however, differ from *M. subterraneus* in having polyunsaturated C_{16} fatty acids and rather smaller amounts of $C_{20:5}$. The composition of the fatty acids derived from the sterol esters of the three algae is shown in Table 5 and is characterized by the lower proportions of C_{16} fatty acids and the presence of saturated, long chain, odd- and even-numbered fatty acids.

DISCUSSION

The three species of the Xanthophyceae examined have two main sterols, cholesterol and clionasterol. No other 4-demethylsterols were detected but this does not rule out the presence of others, as minor components, which would only become apparent if larger quantities of lipid were examined. It is significant that clionasterol has the β-configuration at C-24 (24*S*); in this respect, therefore, the Xanthophyceae are like the Chrysophyceae

TABLE 5. COMPOSITION OF THE FATTY ACIDS OBTAINED AFTER SAPONIFICATION OF THE STEROL ESTERS OF *Botrydium granulatum*, *Tribonema aequale* AND *Monodus subterraneus*

Fatty* acid	*Botrydium* 14 day	*Tribonema* 14 day	*Monodus* 8 day	*Monodus* 11 day	*Monodus* 14 day
	Percentage compostion				
12:0	0·9	2·4	0·3	0·1	0·2
14:0	3·2	8·5	2·5	1·2	1·5
14:1	1·6	0·0	0·0	0·0	0·0
15:0	1·5	1·9	1·3	0·5	0·4
16:0	10·8	17·3	10·9	9·4	7·6
16:1	0·9	2·0	2·0	1·2	2·0
17:0	2·4	3·2	1·6	2·3	3·4
18:0	4·6	1·2	3·3	5·6	7·7
18:1	8·0	9·0	5·6	5·1	4·4
18:2	0·0	0·6	3·7	0·8	2·3
18:3	8·0	17·1	25·1	12·3	13·9
19:0	5·4	3·1	5·0	8·5	11·4
20:0	8·5	4·8	5·8	9·0	10·7
21:0	11·5	7·2	9·8	13·4	11·9
22:0	11·7	7·4	7·2	10·0	8·4
23:0	12·1	9·0	9·4	10·4	8·2
24:0	8·8	5·2	6·7	10·6	6·0

The fatty acids were analysed as their methyl esters on either 10% SP 1000 or 10% FFAP.

* The number before the colon is the number of carbon atoms in the fatty acid; the number after the colon is the number of double bonds present.

Monodus was grown on Bold's Basal Medium with forced aeration; *Botrydium* and *Tribonema* were grown in shake culture on the same medium.

and the Chlorophyta in synthesizing 24*S*-sterols. The two 4,4-dimethylsterols, cycloartenol and 24-methylenecycloartenol, present in small amounts in the three species of Xanthophyceae have also been detected in two members of the Chrysophyceae, *O. danica* and *O. malhamensis.*[24]

It is apparent that the ratio of the major sterols in *T. aequale* and *M. subterraneus* does not change as the culture ages from 8 to 14 days; growth curves showed that 8, 11 and 14 day cultures represented early, mid and late logarithmic phases of growth. However, a change in cultural conditions, from lesser to greater aeration of the medium, caused an increase in the percentage of cholesterol in *M. subterraneus*. Such variations in sterol composition have been seen in single species of Rhodophyta.[7] The sterol composition of the sterol esters largely reflects the total sterol composition and it is unlikely that any great significance attaches to the minor variations seen.

There was, however, quite a difference between the fatty acid composition of the sterol esters and that of the total lipid. In each of the three algae the percentage of C_{16} fatty acids dropped markedly and that of the $C_{18:3}$ and $> C_{20}$ fatty acids rose equally markedly. This indicates that the composition of the fatty acyl moieties of sterol esters is different from those of triglycerides and phospholipids in these alga and suggests that random esterification is not taking place. This phenomenon has been seen in the fungus *Phycomyces blakesleeanus*[27] and may be explained by there being different pools of fatty acid within

[27] BARTLETT, K. and MERCER, E. I. (1974) *Phytochemistry* **13**, to be published.

the organisms or by some of the enzymes taking part in the formation of these lipids exerting a degree of specificity with respect to fatty acids or fatty acid-containing substrates.

EXPERIMENTAL

Organisms and cultural conditions. Botrydium granulatum L. Greville 805/3a Vischer, *Tribonema aequale* Pascher and *Monodus subterraneus* Peterson 848/1 Lewin, U.S.A. were obtained from the Culture Collection of Algae and Protozoa, The Botany School, Cambridge. All three algae were normally grown on Bold's Basal Medium[28] at 23° on a gyrotary shaker under constant illumination (3750 lx) from "warm white" fluorescent tubes. However, *M. subterraneus* was also grown on Bold's Basal Medium contained in Roux bottles standing upright 15 cm from a double bank of "warm white" fluorescent tubes (4750 lx) and vigorously and continuously aerated; these conditions provided a temp. of 20–21°.

Extraction of lipid. The algae were harvested by centrifugation. The cells of *B. granulatum* were broken by passing them through a French Pressure cell 2 × and then extracted 3 × with boiling MeOH, then 3 × with Me_2CO and finally 3 × with Et_2O. The extracts were bulked and mixed with an equal vol. Et_2O. Water was then added until two phases were produced. The ethereal phase was run off and washed with H_2O several times. The aqueous phase was re-extracted several times with Et_2O, the extracts bulked, washed with H_2O, combined with the original ethereal extract, dried over anhyd. Na_2SO_4 and evaporated to dryness under N_2. The filaments of *T. aequale* and the cells of *M. subterraneus* were not susceptible to disruption in the French Pressure Cell and so were thoroughly ground up with washed silver sand in the presence of hot MeOH. The methanolic extract was filtered off and the residue re-extracted, after further grinding, 2 × more with hot MeOH, then 3 × with Me_2CO and finally 3 × with Et_2O. The extracts were bulked and the lipid isolated by the procedure described above.

Saponification procedure. Lipid and sterol esters were refluxed for 1 hr in 6% (w/v) KOH in 90% (*v/v*) aq. EtOH containing 0·25% (*w/v*) pyrogallol as an antioxidant. The saponification mixture was then cooled, diluted with 4 vol. H_2O and the unsaponifiable lipid or sterol removed by repeated extraction with Et_2O. The residual saponification mixture was then acidified to pH 1 with HCl and re-extracted with Et_2O to obtain fatty acids.

Column chromatography. Unsaponifiable lipid was chromatographed on columns of acid-washed, Brockmann Grade 3 alumina (Woelm) developed in a stepwise manner with successive vols of petrol., 2, 4, 6, 8, 10, 12 and 20% Et_2O in petrol. A sterol ester-containing fraction was obtained from the total lipid by chromatography on the same type of column developed initially with petrol. and then with 2% Et_2O in petrol.: the latter solvent eluted all the sterol esters but no unsterified sterols.

TLC. System 1: silica gel G (0·25 mm) impregnated with Rhodamine 6G[29] developed with $CHCl_3$. System 2: silica gel G (0·25 mm) impregnated with Rhodamine 6G developed with C_6H_6–petrol. (2:3).

GLC. Sterols were analysed on 183 cm × 4 mm i.d. glass columns packed with 3% OV-1 on 100–120 mesh Gas Chrom Q operated isothermally at 240° using argon, flowing at 40 ml/min, as the carrier gas. Detection was by FID. Cholestane was chromatographed with each sample and retention times were determined relative to cholestane. GC–MS of sterol mixtures was carried out on a Pye 104 gas chromatograph fitted with a 213 cm × 4 mm i.d. glass column packed with 1% SE-52 on 100–120 mesh Gas Chrom Q at 240° combined with an AEI MS-30 mass spectrometer. Fatty acid methyl esters were analysed on 183 cm × 4 mm i.d. glass columns packed with either 10% SP 1000 on 100–120 mesh Chromosorb WAW or 10% FFAP on 80–100 mesh Chromosorb 101. Dual column temp. programming was used; the initial temp. was 160° rising at 7·5°/min to a final temp. of 240° which was then held. The carrier gas was argon flowing at 40 ml/min and detection was by FID.

Preparation of fatty acid methyl esters. This was accomplished in the usual manner with the boron trichloride–MeOH reagent.

Hydrogenation of fatty acid methyl esters. This was carried out by shaking a solution of the fatty acid methyl esters in ethyl acetate in an atmosphere of H_2 in the presence of Adam's catalyst.

Optical rotation measurement. The specific rotation $[\alpha]_D^{25}$ of sterols was determined in $CHCl_3$ with a Bellingham and Stanley Polarmatic 62 Spectropolarimeter.

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[28] DEASON, T. R. and BOLD, H. C. (1960) *University of Texas Publication* No. 6022, 1.
[29] AVIGAN, J., GOODMAN, D. S. and STEINBERG, D. (1963) *J. Lipid Res.* **4**, 100.